Rev. Int. Contam. Ambie. 32 (1) 101-117, 2016

RELATIONSHIP BETWEEN GENOTOXIC DAMAGE AND ARSENIC BLOOD CONCENTRATIONS IN INDIVIDUALS RESIDING IN AN ARSENIC CONTAMINATED AREA IN MORELOS, MEXICO

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(Recibido febrero 2015; aceptado mayo 2015)

Key words: biomarkers, human exposure, genetic damage, comet assay, chromosome aberrations

ABSTRACT

Arsenic (As) contaminated drinking water is a well-known problem that still affects millions of people worldwide. Therefore, biomonitoring studies of human populations exposed to arsenic via drinking water along with the search for new biomarkers become important. Huautla, Morelos, Mexico, is a mining district where 780 000 tons of toxic wastes have been discharged at 500 m from Huautla town, where the main contaminants are Pb and As and still, there is no information about their effect on the human population health. Therefore, the aims of this study were: A) To examine As concentration in drinking water and in whole blood samples from the individuals residing in Huautla, Morelos, B) To evaluate DNA damage levels in whole blood lymphocytes from the exposed individuals and C) To evaluate if there is a correlation between DNA damage and total As blood levels from the exposed individuals. Our results demonstrate that drinking water from Huautla, Morelos, is contaminated with As (0.240 ± 0.008 µg/ml) exceeding the national (0.025 µg/ml) and international standards (0.010 µg/ml). Total As levels in whole blood samples from the exposed individuals corroborate this exposure (60 ± 9 µg/l). As stimulated positively the lymphocyte cell cycle and induced DNA breaks and chromosome aberrations (CA), which were positively and significantly correlated with As concentrations in whole blood samples. Among the types of CA analyzed, terminal deletions registered the highest determination coefficient ($R^2 = 0.70$). We provide evidence that supports the use of total As blood concentration as an internal biomarker of exposure in human populations. A fact that might be of particular interest in subjects with kidney related conditions, in which the normalization of total As/gr creatinine in urine may not reflect accurately the exposure level to this metalloid.

Palabras clave: biomarcadores, exposición humana, daño genético, ensayo cometa, aberraciones cromosómicas

RESUMEN

La contaminación por arsénico (As) en el agua es un problema conocido que sigue afectando a millones de personas en el mundo. Por lo que, el biomonitoreo de poblaciones humanas expuestas a As a través del agua destinada al consumo humano en conjunto con la búsqueda de nuevos biomarcadores es fundamental. Huautla, Morelos, México, fue un distrito minero, en el cual existen 780 000 ton de residuos mineros localizados a 500 m de la población y en donde los principales contaminantes son el Pb y el As y aún no hay información relacionada a sus efectos sobre la salud de la población. Los objetivos del presente trabajo fueron: A) Examinar las concentraciones del As en el agua y en muestras de sangre entera de individuos de la población de Huautla, Morelos, B) Evaluar los niveles de daño al ADN en linfocitos de sangre periférica de la población expuesta y C) Evaluar si existe una correlación entre los niveles de daño al ADN y la concentración de As en sangre de los individuos expuestos. Los resultados obtenidos demuestran que el agua para beber de la localidad de Huautla está contaminada por As (0.240 ± 0.008 μg/ml) excediendo las normas nacionales (0.025 μg/ml) e internacionales (0.010 μg/ml). La concentración de As en las muestras de sangre entera de los individuos expuestos corrobora la exposición (60 ± 9 μg/L). El As afectó el ciclo celular e indujo rompimientos en el ADN, así como aberraciones cromosómicas (AC), las cuales se correlacionaron positiva y significativamente con la concentración de As en sangre. Entre las AC analizadas, las deleciones terminales fueron las que registraron el coeficiente de determinación más alto ($R^2 = 0.70$). Este trabajo provee evidencia que apoya el uso de la concentración total de As en sangre como biomarcador de exposición interna en poblaciones humanas. Lo que resulta importante en particular en individuos con enfermedades relacionadas con disfunción renal en los cuales la normalización de As/gr creatinina en orina puede no reflejar correctamente el nivel de exposición al metaloide.

INTRODUCTION

Exposure to high levels of arsenic in drinking water has been documented for many years in various regions of the world. Millions of people are at risk of developing cancer and other diseases because of chronic arsenic (As) exposure (Florea *et al*. 2005, Tsuji *et al*. 2014).

Arsenic is known to be a potent human carcinogen, classified by the International Agency for Research on Cancer in group 1A (IARC 2004). The proposed mechanisms underlying its carcinogenicity are: activation of cell cycle in different cell types, induction of chromosomal aberrations and aneuploidy, oxidative stress and interference with DNA repair enzymes (Kitchin and Ahmad 2003, Wnek *et al*. 2011). In its organic form, As is known to be cytotoxic and genotoxic *in vivo* and *in vitro* (Dopp *et al*. 2004), causing clastogenic effects such as single strand breaks, formation of apurinic/apyrimidinic sites and DNA-protein crosslinks (Sordo *et al*. 2001, Salazar *et al*. 2009, Wnek *et al*. 2011).

In order to assess the levels of exposure to this element in human populations, various biomarkers have been developed such as total urinary As, hair and toenail As. Toenail As reflects past As exposure (Karagas *et al*. 2000), nail As reflects several months of exposure but not recent exposure (Wu *et al*. 2001) and it may have limited utility in highly exposed populations (Schmitt *et al*. 2005, Hall *et al*. 2007). Total urinary arsenic has been widely used in exposed populations. However, in subjects with kidney related conditions who have high creatinine levels in urine, the measurement and normalization of As would be difficult. Recently, blood As levels have been purposed as a good biomarker of internal dose (Gamble *et al*. 2007, Hall *et al*. 2007). Still, little is known about the relationship between blood As levels in chronic exposed populations and the induction of different genotoxic endpoints. To assess the genotoxicity of several chemical agents, the alkaline single cell gel electrophoresis or "comet assay" is a sensitive and reliable method for detecting alkali-labile and transient repair gaps measured as DNA single strand breaks (SSB) in eukaryotic individual cells. The comet assay has been considered as an early biomarker of effect, widely used to assess DNA damage in several population studies where people were exposed to genotoxins whether occupationally or environmentally

(Collins *et al*. 2008, Mussali *et al*. 2013). The frequency of chromosome aberrations (CA) in peripheral blood lymphocytes has been long used as a biomarker of effect for many human carcinogens. Assuming that the formation of CA is similar in different tissues, the level of damage in lymphocytes can be expected to reflect the level of damage in cancer prone tissues and to indicate risk of cancer and other chromosome instability associated diseases (Bonassi *et al*. 2000, Bonassi *et al*. 2004, Norppa *et al*. 2006). In populations chronically exposed to arsenic via drinking water, numerous studies have documented an association between the induction of CAs and As levels in drinking water and urine (Basu *et al*. 2005, Norppa *et al*. 2006) but none has investigated this association with total As blood levels during environmental exposures. A fact that becomes necessary, specially in individuals with altered kidney function, as chronic diseases such as Type 2 diabetes mellitus and hypertension. Human exposures to inorganic As have been linked to an increased risk of diabetes mellitus (Del Razo *et al*. 2011, Currier *et al*. 2014). Under these conditions, the clearance of As from the body via the kidneys, may not reflect accurately the exposure to the metalloid, also the normalization of total urinary As per gram of creatinine runs the risk of confounding relationships between total urinary As and As metabolism (Gamble *et al*. 2005).

In Mexico, As levels in drinking water have been found to exceed the international standards set by the World Health Organization (WHO 2006) of 0.010 mg/L and by the Mexican standard, NOM-127-SSA1-1994 of 0.050 mg/L in the year 2000. This standard was modified to reach a maximum permissible limit of 0.025 mg/L by the year 2005.

Various states in Mexico are known to be arsenic "hyperendemic", an example of this problem can be found at the southern part of Morelos state, specifically at the municipality of Tlaquiltenango, where Huautla town is located. This place is recognized for its historic mining activity since the XVI century until 1992, specially lead (Pb), silver (Ag) and zinc (Zn).

There used to be four mines in the region, none of them are currently active. Nevertheless, tons of toxic wastes have been discharged in the area. It has been estimated that there are about 780 thousand tons of mine wastes, and the majority of them are rich in Pb, manganese (Mn) and cadmium (Cd) that haven't been processed or neutralized. As a consequence, several mine tailings were left behind in the area, containing high Pb and As concentrations (2298 and 139 mg/kg, respectively). Therefore, it is estimated that the principal contaminants of soil and groundwater are As and Pb (SEMARNAT 2004, 2005). Recent studies reported that concentrations of several metals -including As- were statistically higher in a group of small mammals living inside Huautla mine tailings than in an unexposed group. The exposed group had the highest levels of DNA damage (Tovar-Sánchez *et al*. 2012) along with the lowest population densities, lowest levels of genetic diversity and high endogamy (Mussali-Galante *et al*. 2013).

The latter scenario along with the high As content in Morelos state's natural resources such as arsenopyrite, scorodite and orpiment, may contribute to a hazardous exposure to contamination on Huautla inhabitant's health. Also, Huautla has a water distribution network that comes directly from inside the mine "Pájaro Verde" to a storage tank. Huautla residents have been using this water as the main source of drinking water since the mine was closed in 1992. In spite of this, there are no studies assessing the genotoxic effects of metal contaminated drinking water on the individuals living in this region.

Mexico is considered one of the 10 countries with the largest numbers of people with diabetes (Shaw *et al*. 2010) and the majority of Huautla settlers have been diagnosed with type 2 diabetes mellitus and hypertension, two of the most common diseases that affect kidney function that results in high creatinine levels (Bakris *et al*. 2000).

The aims of this study were A) To examine As concentration in drinking water (mine and storage tank) and in whole blood samples from individuals residing in Huautla, Morelos, B) To evaluate DNA damage levels in lymphocytes from the exposed individuals and C) To evaluate if there is a correlation between DNA damage induction and total As blood levels from the exposed individuals.

MATERIALS AND METHODS

Informed consent form

Written informed consent was obtained from each volunteer participating in the study.

This project was evaluated and approved by the Ethics and Scientific Committee from the Faculty of Medicine at the National Autonomous University of Mexico (UNAM).

Questionnaire

Each volunteer filled a pre-designed questionnaire with general data, and other information such as time of residence, use of ground water, water-bore

length, disease incidence, reproductive and family histories (up to three generations), alcohol intake, and smoking status.

Inclusion criteria for volunteers

The volunteers participating in this study were males ranging from 20 to 70 years old. A total of 22 participants were included in the study, which were long-term residents or were born there. All the participants in this study were peasants with the following characteristics:

1 Not previous history of cancer.
2 Not a previous history of having an infectious disease three months earlier to the date of sampling.
3 Not being under any medical treatment three months earlier to the date of sampling.
4 Not practicing a medical exam involving radiation of any type 3 months earlier to the date of sampling.
5 Volunteers should take drinking water from the water distribution network that comes directly from inside the mine "Pájaro Verde".

Exposed population

This study was conducted in Huautla, Morelos, Mexico. This zone is located at the southern part of Morelos state in the municipality of Tlaquiltenango (18º25′ N – 99º01′ W). Huautla is located inside a protected natural reserve known as the "Reserva de la Biosfera Sierra de Huautla (REBIOSH)" (SEMARNAT 2004, Dorado *et al*. 2005), that was also decreed as "Patrimony of the humanity" by the UNESCO in 1996. As a result of mining activities three tailings were left in this zone in open air and without any environmental care. The main impoundment (18º26′ N – 99º01′ W, 995 masl) contains high Pb and As concentrations (2298 and 139 mg/kg, respectively) and it is located at approximately 500 m from Huautla town (SEMARNAT 2004, INEGI 2009). These residues were left near lakes that flow into the Amacuzac river. Hence, there are possibilities that during rainy season, these residues may lixiviate, leach, or run off to other zones (SEMARNAT 2004, 2005). Additionally, Huautla has a water distribution network that comes directly from inside the mine "Pájaro Verde", the water is then carried to a storage tank of a 55m^3 capacity through a 4 inch diameter pipe with an approximate length of 2 km. This water is disinfected with sodium hypochlorite twice a year and then it is distributed to the households. Out of the 22 volunteers that participated in this study, 18 were previously diagnosed with hypertension (82 % of the studied population) and 16 were previously diagnosed with type 2 diabetes mellitus (73 %). Previous studies on this same individuals reported that the mean concentration (mean ± s.d.) of creatinine in spot urine samples was 3.802 ± 1.31 g/L (range, 0.63 - 6.0 g/L).

Reference population

Reference population was chosen in order to match age, socio-economic status and lifestyle of the exposed population. A total of twenty participants were included in the study. Reference individuals come from the town of Ajuchitlán which is also located inside the "REBIOSH". It is located southwest of the municipality of Tlaquiltenango (N - 18º 27' 48" W - 98º 58' 22", 1.060 masl) (INEGI 2009).

Ajuchitlán drinking water networks are completely independent and different from the Huautla drinking water networks. There are no mines near the area and there are no records of any possible contamination by metals in the zone.

Ajuchitlán has three water wells which the population uses as the main sources for drinking water, these are: "well 1", "well 2" and "well 3". These water wells are disinfected with sodium hypochlorite twice a year and then, the water is distributed to the households.

Drinking water samples

For the reference group, a total of 36 samples were taken from three different water wells (six in dry and six in rainy season for each well). A total of 24 water samples were taken from the exposed population. For the exposed population, 12 samples were taken from inside the mine "Pájaro Verde" and 12 from the storage tank (six in dry and six in rainy season). The sampling methods were conducted according to the Mexican standards for drinking water sampling NOM-127-SSA-1994. The samples were transported on ice to the lab in plastic containers with 2 mL of HNO_3 ultrapure (J.T. Baker 6901-5).

Blood samples

A total of 22 blood samples were taken from Huautla individuals by vein puncture in K_2EDTA vacutainer tubes and transported on ice to the laboratory. They were processed immediately by the Single Cell Gel Electrophoresis **(**SCGE) assay, lymphocyte cultures and metal determination. Blood samples were similarly collected from age, sex and socio-economically-matched control individuals (n = 20). The same blood sample was used to analyze genetic damage (chromosomal aberrations and single strand breaks).

Metal determination in drinking water and whole blood samples

Metal concentrations in water and blood samples were determined by atomic absorption spectrophotometer. A Perkin Elmer model 3100 equipped with a graphite furnace HGA600 for Pb and Cd was used. A Perkin Elmer equipped with flame 2380 was used for Zn and Cu determinations. A Perkin Elmer model 3100 equipped with a hydride generator MHS-10 was used to determine total As concentrations.

Single cell gel electrophoresis assay

The alkaline SCGE assay was performed as described by Tice *et al*. (1992). Twenty µL of blood were obtained and mixed with 75 µL of 0.5 % of low melting point agarose, 75 µL of this mixture was pippeted onto a slide previously covered with 170 µL of agarose and covered with a coverglass to make a microgel on the slide. Slides were kept in an ice-cold tray to allow the agarose to gel. The coverglass was removed, and 75 µL of agarose was layered as before. Slides were immersed in lysing solution (2.5 M NaCl, 100 mM Na_2EDTA, and 10 mM Tris-base, pH 10, 4 ˚C). After lysis, for at least 1 h, slides were placed on horizontal electrophoresis. The DNA was allowed to unwind for 20 min in electrophoresis running buffer solution (300 mM NaOH and 1 mM Na_2EDTA, pH> 13). Electrophoresis was conducted for 20 min at 300 mA at a voltage of 1 V/cm. All technical steps were conducted by using very dim indirect light. After electrophoresis, the slides were removed, and alkaline pH was neutralized with 0.4 M Tris, pH 7.5. The slides were dehydrated in three steps with absolute ethanol for 5 min each. Ethidium bromide (75 mL of a 20 mg/mL solution) was added to each slide and a coverglass was placed on the gel. DNA migration was analyzed on Olympus BMX-60 microscope with fluorescence equipment and measured with a scaled ocular as the tail length. For the evaluation of DNA migration, 100 cells were scored for each individual.

All chemical reagents were purchased from Sigma Chemicals Company, St. Louis, MO, USA.

Lymphocyte culture for the evaluation of chromosome aberrations

Whole blood samples were cultivated in a Roswell Park Memorial Institute-medium (RPMI-1640 medium; Sigma Chemical Co. St. Louis, MO, USA) at 37 ºC and 5 % of CO_2 in the presence of 0.5 mL of phytohemagglutinin (Roche Diagnostics), 20 % fetal bovine serum (Biological Industries, Israel), 5000 IU/mL penicillin and 1000 IU/mL streptomycin. Four lymphocyte cultures were done per donor.

In order to performe the analysis of chromosomal aberrations, lymphocytes were grown for a total of 48 h in the presence of 4 µg/mL of colchicine (Sigma chemical Co. St. Louis, MO, USA) for the last 3 h. Thereafter, the cells were treated with hypotonic solution (0.075 M KCI) and then fixed in acetic acid-methanol (1:3). Metaphase spreads were prepared and the slides were processed for Giemsa staining, employing standard protocols. At least 100 metaphases were analyzed for each subject. The analysis of structural aberrations included: acentric fragments, isochromatidic fragments, terminal deletions and gaps. Cells containing any of these types of chromosomal alterations were considered aberrant cells.

Mitotic (MI) and replication indexes (RI)

For the analysis of mitotic and replication indexes, 24 h after lymphocyte harvest, 5-bromo-deoxiuridine (5 µg/ml) was added to two cultures of each donor and were incubated for another 48 h at 37 ˚C with 5 % of CO_2 in the presence of 4 µg/mL of colchicine for the last 3 h.

After 48 h (MI) or 72 h (RI) of lymphocyte culture, the cells were incubated at 37 ˚C for 15 min with hypotonic solution (0.075 M KCI) and then they were fixed in acetic acid-methanol (1:3). For the analysis of mitotic index, metaphase spreads were prepared and the slides were processed for Giemsa staining, employing standard protocols. For replication index analyses: Before Giemsa staining, glass slides containing the cell spreads were stained with Hoechst H-33258 fluorochrome for 20 min, incubated in standard saline citrate (SSC) solution (SSC 2X) and irradiated with UV light for 20 min. Finally, slides were incubated at for 1 h at 60 ˚C with SSC 2X, air dried and stained with Giemsa. This method identifies cells which have performed different number of DNA replications in culture. Moreover this system can indicate the proportion of cells that are in mitosis at a given time (Perry and Wolf 1974). All the above reagents were purchased from Sigma Chemical Co. St. Louis, MO, USA.

Microscopic analysis was performed to determine the MI, scoring the number of metaphases in 2000 stimulated nuclei (Rojas and Valverde 2007).

Cell proliferation kinetics was evaluated on 100 consecutive metaphases determining the number of first (M1) second (M2) and third (M3) division cycles. The RI was calculated using the following formula.

$$RI = [M1 + 2(M2) + 3(M3)] / 100.$$

DATA ANALYSIS

Comet assay

Nested analysis of variance (ANOVA) was conducted to test differences for As water concentration between water bodies, seasons and for the interaction tank × season.

One-way ANOVA was performed to determine the effect of As blood concentration for both populations studied (reference *vs*. exposed) on DNA damage induction determined by the alkaline comet assay in whole blood lymphocytes.

Subsequently, a Tukey analysis was conducted to determine differences between the levels of DNA damage of the reference population and the mean DNA damage of each individual of the exposed population.

Regression analysis was used to test the relationship between As blood concentration and genotoxic damage. Also, regression analysis was performed separately for each population studied in order to determine the relationship between smoking status, drinking habits, and age on the level of genotoxic damage.

Chromosome aberrations

A *t*-student test was done in order to know if there was an effect of metal exposure on the MI and RI of whole blood lymphocytes for both the exposed and the reference populations**.** For the evaluation of CAs, all the data (percentage) were transformed as X = arcsin (%)½ (Zar 2010). We used the Shapiro-Wilk "*W*" test which is used to probe normality. The results reported show that W test was not significant in all cases (acentric fragments $W = 0.932$, $P = 0.10971$; Gaps $W = 0.96059$, $P = 0.45040$; Terminal deletions $W = 0.91198$, $P = 0.22618$; Isochromatidic fragments $W = 0.93224$, $P = 0.10939$, Total number of chromosome aberrations (T.N.C.A.) with gaps $W = 0.95072$, $P = 0.28087$; T.N.C.A. without gaps $W = 0.92548$, $P = 0.06444$; aberrant cells with gaps $W = 0.96441$, $P = 0.53324$; aberrant cells without gaps $W = 0.92718$, $P = 0.08431$). Hence, the hypothesis that the respective distribution is normal was accepted (Zar 2010). We performed *t*-student tests to determine if there was an effect of As levels in drinking water and total As blood levels on all the CAs analyzed (Zar 2010).

Regression analyses were conducted in order to test the relationship between each type of CA studied and As blood concentrations from the exposed and reference populations. Data were log transformed ($X' = \log X+1$; Zar 2010).

Finally, regression analyses were conducted in order to know the relationship between smoking status, drinking habits, and age of the exposed and reference individuals on total number of CA studied with and without gaps**.** Data were also transformed ($X' = \log X+1$; Zar 2010) to analyze the frequency of the total number of CA, statistical analyses were done with and without "gaps"

RESULTS AND DISCUSSION

Metal concentrations in drinking water

Metal concentrations in drinking water samples from the control and exposed groups are shown in **table I**. The results clearly indicate that all the metals examined in drinking water samples from the control and exposed groups do not exceed the maximum permissible levels in any of the water bodies examined, in accordance to the Mexican standard NOM-127-SSA1-1994, for Pb (0.01 µg/ml), Cu (2.0 µg/ml), Zn (5.0 µg/ml), Cd (0.005 µg/ml). The latter with exception of As, which levels were highly increased both in the mine and in the storage tank of the exposed population. According to the Mexican standard NOM-127-SSA1-1999, As levels in drinking water

TABLE I. MEAN METAL CONCENTRATIONS (µg/mL; MEAN ± STANDARD ERROR) IN DRINKING WATER SAMPLES FROM THE REFERENCE AND THE EXPOSED POPULATIONS

Metal	Reference			Exposed		
	Well 1	Well 2	Well 3	Mine	Storage tank	MPL
Pb	N.D.	N.D.	N.D.	N.D.	N.D.	0.01
As	N.D.	N.D.	N.D.	0.230 ± 0.032	0.240 ± 0.008	0.025
Cu	N.D.	N.D.	N.D.	N.D.	N.D.	2.00
Zn	0.08 ± 0.018	0.03 ± 0.011	0.03 ± 0.005	0.02 ± 0.007	0.04 ± 0.005	5.00
Cd	N.D.	N.D.	N.D.	N.D.	N.D.	0.005

Pb (Lead), As (Arsenic), Cu (Copper), Zn (Zinc), Cd (Cadmium), N.D. (not detected), MPL (maximum permissible level)

should not exceed 0.025 mg/L. Hence, As levels in the storage tank were 8 fold higher, and according to the international standards (EPA 2001, FAO 2006) they were 19.5 times higher. On the other hand, the mine registered nine times higher levels of As according to the Mexican standard and 22.5 times higher according to the international standards (**Table I**). In general, As concentration in drinking water did not differed significantly between the mine and the storage tank ($F = _{1,3819}$, $P > 0.05$), seasons ($F = _{1,2637}$, $P > 0.05$), and between the interaction tank × season ($F = _{1,1970}$, $P > 0.05$).

These results demonstrate that drinking water from Huautla, Morelos is contaminated with As. The presence of As in drinking water might be the result of mine tailings in the zone that were left in open air and near a series of lakes that disembogues at the Amacuzac river. These residues may be lixiviating into the ground water. In addition, Morelos state soils have minerals with high As content such as arsenopyrite, scorodite and orpiment that could also contribute to this problem. The results obtained in the present study and by the SEMARNAT (2004, 2005), indicate that Huautla tailings have a high As content (274 mg/kg), exceeding the maximum permissible levels set by national standards (NOM-127-SSA1-1994) (residential soils 20 mg/kg and industrial soils 40 mg/kg), we conclude that the high As concentrations registered inside the mine and in the storage tank may be the result of natural and anthropogenic activities.

Arsenic blood concentrations in reference and exposed individuals

Metal blood concentrations of the reference and exposed individuals demonstrated that Pb, Cu, Cd and Zn are below the maximum permissible levels for adult individuals not occupationally exposed (Majid *et al*. 1999, NOM-199-SSA-2000, Ramírez 2006; **Table II**). In contrast, exposed individuals registered a mean As whole blood concentration of 60 ± 9 µg/L (range 20-200 µg/L; **Table II**).

In this study, we evidenced that Huautla inhabitants are exposed to As via drinking water and we also corroborate this exposure with internal As whole blood concentrations. Exposed individuals registered a mean As whole blood concentration of 60 µg/L, which are above the limit suggested by the ATSDR (2007), Hall *et al*. (2006, 2007) and Gamble *et al*. (2007), who established that this metalloid should not exceed 50 µg/L in peripheral blood. During single exposures, As blood concentrations are rapidly cleared from the body by the kidneys, so these concentrations would reflect only recent exposures

TABLE II. MEAN METAL CONCENTRATIONS (µg/L; MEAN ± STANDARD ERROR) IN WHOLE BLOOD SAMPLES FROM THE REFERENCE AND THE EXPOSED POPULATIONS

Metal MPL	Pb 200	Cu 1000	Zn 6600	Cd 500	As 50
Reference N = 20	71 ± 10	987 ± 32	6432 ± 245	N.D.	N.D.
Exposed N = 22	60 ± 10	870 ± 30	6420 ± 310	N.D.	60 ± 9

Pb (Lead), As (Arsenic), Cu (Copper), Zn (Zinc), Cd (Cadmium), N.D. (not detected), MPL (maximum permissible level)

(Pandey *et al*. 2007). However, during chronic and continuous exposures, steady state concentrations of the metalloid are achieved, reflecting past exposures and the load of As in the individual. Therefore, it has been used as a biomarker of past exposures (Morton and Dunette 1994, Wu *et al*. 2001, Gamble *et al*. 2007, Pandey *et al*. 2007, Marchiset-Ferlay *et al*. 2012). Moreover, when chronic exposures occur, As concentrations of different tissues may be released into the hematopoietic system, a fact that may reflect the total As load in the body (Morton and Dunette 1994, Wu *et al*. 2001, Hall *et al*. 2006, Gamble *et al*. 2007). Also, when analyzing total As blood concentrations, high inter-individual differences have been reported (Vuyuri *et al*. 2006), as in the case of our study (from 20 to 200 µg/L). These differences may be attributed to individual susceptibility factors (arsenic metabolism, polymorphism of several activating/detoxifying enzymes), lifestyle factors and nutritional status (Chen *et al*. 2005, Ahsan *et al*. 2006, Ahsan *et al*. 2007, Martínez *et al*. 2011).

Total As blood concentrations have been correlated with other variables such as, As concentrations in drinking water, time of exposure and total urinary As levels (Morton and Dunette 1994, Goyer *et al*. 1999, Klassen 2001, Mandal *et al*. 2007), suggesting that measurements of As blood concentrations are a suitable biomarker to study chronic human exposures.

In this context, Hall *et al*. (2006) studied a human population chronically exposed to As via drinking water, they found that when drinking water As levels increased as well as time of exposure, As blood concentrations of these individuals also increased. They reported a positive and significant relationship between the levels of As in drinking water and total As blood concentrations in exposed individuals.

Wu *et al*. (2001) reported a positive and significant relationship between As blood levels and the

level of reactive oxygen species, and a negative and significant relationship between As blood levels and antioxidant plasma levels in the same subjects. Additionally, Vahter *et al*. (1995) analyzed a human population exposed to the metalloid, reporting As drinking water levels of 0.200 mg/L and a mean As blood concentration of 76 µg/L in Andean women, these results are in agreement with our observations (As water levels: 0.237 mg/L, As blood concentration: 60 µg/L). Therefore, we suggest that total blood As concentrations of exposed individuals are a suitable biomarker of internal exposure, that is useful for studying chronic exposures, that offers information about total As body burden and that is well correlated with other biological variables such as: As concentrations in urine and drinking water (Morton and Dunette 1994, Klassen 2001, Hall *et al*. 2006, Gamble *et al*. 2007, Pandey *et al*. 2007), plasma levels of oxidants and antioxidants (Wu *et al*. 2001) genetic expression levels of pro-inflammatory molecules in whole blood lymphocytes (Wu *et al*. 2003) and vascular diseases (Wu *et al*. 2003, Tchounwou *et al*. 2004, Pandey *et al*. 2007), among others.

Mitotic and replication indexes

MI values from lymphocytes of the reference population (mean ± s.e.) 4.95 ± 0.63, were statistically different from the MI values of the exposed population 7.44 ± 0.58. (*t*- student, $P < 0.01$). Also, RI values were statistically different from the reference population (1.37 ± 0.05) and the exposed population (1.96 ± 0.06; *t*-student, $P < 0.001$). These results showed that whole blood lymphocytes from the exposed individuals respond faster to the proliferation stimulus (phytohemaglutinine) than lymphocytes from the reference individuals, resulting in an increasing number of metaphase cells in the exposed individuals (**Fig. 1**). Moreover, lymphocyte proliferation kinetics of the exposed individuals indicated that they cycle faster than the lymphocytes from the reference population. These results can be explained by the fact that As can induce cell cycle activation in many ways.

It has been suggested that As at chronic, low and non-toxic concentrations (0.1 µM), alters dependent p53 signaling pathways in human cells, compromising the ability of these cells to respond to genotoxic damage and promoting an increase of p21 (Vogt and Rossman 2001). Also, As may regulate c-Myc positively (Trouba *et al*. 2000), inhibiting the expression of p21 and inducing the expression of cycline-D. Low levels of p21 are required to activate cycline

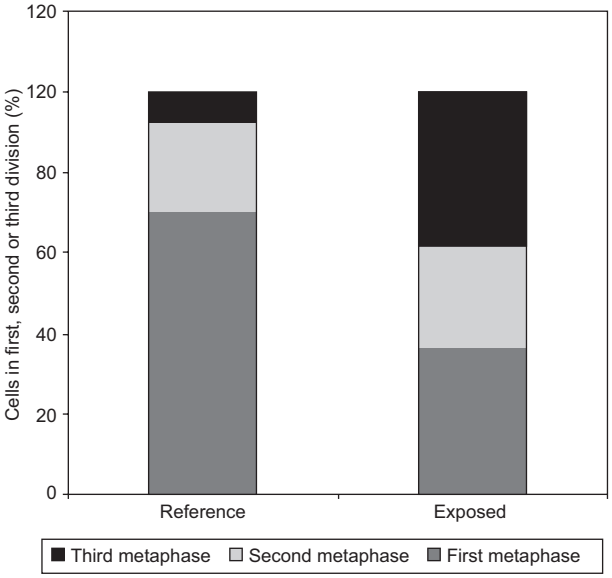

Fig. 1. Percentage of lymphocytes in first, second or third division cycles of the reference and the exposed populations

D1-cdk4/6, whereas high levels of p21 inhibit it (Bouchard *et al*. 1999, Mitchell and El-Deiry 1999, Wen *et al*. 2011). Additionally, when there are low levels of p21 in the cell, pRb phosphorylates and inactivates, inducing positively the cell cycle response. In these cells the p53-p21 apoptotic mechanism is blocked which enhances the activation of the cell cycle**.** It has also been observed that cells with genotoxic damage and an altered p53 are more likely to keep cycling than to commit apoptosis. Hence, these cells may present an elevated frequency of CA (Vogt and Rossman 2001, Wen *et al*. 2011).

Trouba *et al*. (2000) observed that fibroblasts exposed to sodium arsenite registered an increasing number of cells at the "S" phase of the cycle. After analyzing the cell cycle regulatory proteins of the exposed cells, these authors found that survival factors such as c-Myc and E2F-1 were overexpressed, in contrast to factors that regulate negatively the cell cycle such as MAP-K-phosphatase-1 and p27 that were underexpressed. These results support the hypothesis that As deregulates cell cycle kinetics by overexpressing survival factors and by inhibiting other factors that regulate negatively the cell cycle kinetics.

In this study, lymphocytes from the exposed population had higher rates of proliferation than lymphocytes from the reference population in addition to high levels of genotoxic damage registered as SSB and CA. Hence, we suggest that these cells behave as cells with "replicative instability" which is characterized by cells that replicate in the presence

of a great amount of genotoxic lesions such as CA and SSB as a result of exposure to metals and other chemical compounds (Belyaev 2004).

Genotoxicity: Single strand breaks

The results of the DNA damage analysis by the alkaline comet assay in whole blood lymphocytes revealed a statistical significant difference ($F_{1,40}$ = 56.417, $P < 0.001$) in the induction of single strand breaks in exposed individuals compared to the reference group (**Fig. 2**). All individuals from the exposed population also differed significantly from the reference group (**Fig. 3**). A positive and significant relationship between As blood concentration and the induction of SSB was registered ($r = 0.46$, $r^2 = 0.22$, $P < 0.05$).

These results suggest that in this particular population, As induces DNA damage revealed as SSB, confirming the sensitivity and suitability of the comet assay for the analysis of genotoxic damage induced *in-vivo* in human lymphocytes.

These results are in agreement with other studies that report that As in drinking water causes SSB in human lymphocytes in a dose response manner (Yedjou and Tchounwou 2006, Zhang *et al*. 2007).

As a result of reactive oxygen species generation, arsenic can induce DNA adducts and oxidized bases, so when the repairing mechanisms are trying to remove these lesions, specially oxidized bases, apurinic/apirimidinic sites (AP sites) may rise and convert to SSB, which are detected by the comet assay (Guillamet *et al*. 2004, Li *et al*. 2001, Sordo *et al*. 2001, Yedjou and Tchounwou 2006). Another mechanism that may induce SSB is the inhibition of DNA repairing enzymes by As, specifically base excision repair pathways which generates SSB that are detected with the comet assay (Guillamet *et al*. 2004, Yedjou and Tchounwou 2006, Sampayo-Reyes *et al*. 2010).

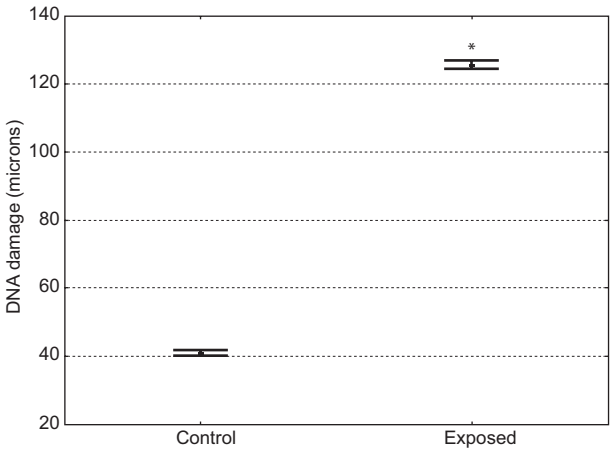

Fig. 2. Differences between the induction of DNA single strand breaks in the reference *vs*. exposed populations. A statistically significant difference (P < 0.001) was registered between both populations (mean ± standard error) on the induction of single strand breaks (SSB) measured with the alkaline comet assay. *(P < 0.001)

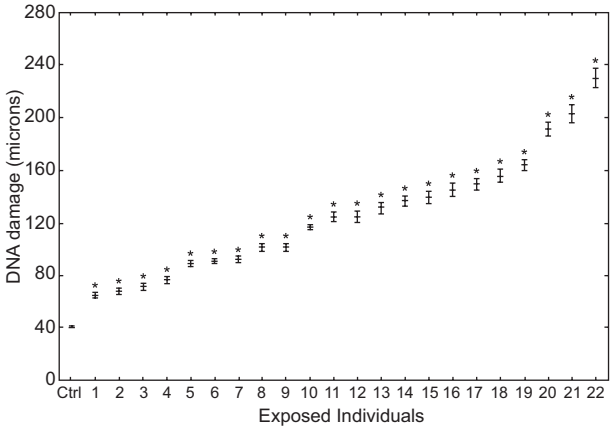

Fig. 3. Differences between the induction of DNA single strand breaks (SSB) in the reference (Control, n = 20) *vs*. each of the exposed individuals. A statistically significant difference (P < 0.001) was registered between the reference group and each of the individuals of the exposed population (mean ± standard error) on the induction of SSB measured with the alkaline comet assay. *(P < 0.001)

We observed that all the individuals of the exposed population differed significantly in the induction of SSB from the control group, even with the individuals that presented As concentrations below 50 µg/L. In this report we indicate that As blood concentrations below this limit, are also genotoxic to human lymphocytes assessed by the alkaline comet assay. There is a positive and significant relationship between As blood concentration and DNA damage. This is another reason to recommend blood As levels as a good biomarker of internal exposure that correlates positively with the induction of SSB.

The levels of DNA damage of the reference group are higher than the historical controls assessed by the comet assay. In this regard, it is important to mention that individuals from the control group are also peasants in contact with pesticides twice a year (the same as the exposed group) and their nutritional status is based on corn, beans and red meat, almost no vegetables are included in their diet. Thus, these factors may contribute to the levels of the SSB registered.

Genotoxicity: Chromosome aberrations

All statistical tests (*t*-student) showed that the frequency of all types of CA analyzed (acentric

TABLE III. MEAN ± STANDARD ERROR OF THE DIFFERENT TYPES OF CHROMOSOME ABERRATIONS ANALYZED IN THE REFERENCE AND THE EXPOSED POPULATIONS. T-STUDENT TEST TO DETERMINE STATISTICALLY SIGNIFICANT DIFFERENCES BETWEEN THE REFERENCE AND EXPOSED POPULATION OF EACH TYPE OF CHROMOSOME ABERRATION ANALYZED

	Reference	Exposed	t_{40}
Acentric fragments	0.017 ± 0.007	0.044 ± 0.007	2.303 *
Gaps	0.030 ± 0.012	0.039 ± 0.007	1.452 ^{ns}
Terminal deletions	0.001 ± 0.000	0.015 ± 0.005	3.239 **
Isochromatidic fragments	0.003 ± 0.003	0.017 ± 0.005	2.116 **
T.N.C.A. with gaps	0.060 ± 0.017	0.115 ± 0.013	2.882 **
T.N.C.A. without gaps	0.030 ± 0.011	0.076 ± 0.010	3.790 **
Aberrant cells with gaps	0.047 ± 0.012	0.975 ± 0.010	2.639 **
Aberrant cells without gaps	0.026 ± 0.009	0.067 ± 0.009	2.621 **

T.N.C.A = Total number of chromosome aberrations. n.s. = not significant. * = $P < 0.05$, ** = $P < 0.001$

fragments, chromatidic deletions, isochromatidic fragments, total number of chromatidic aberrations with or without "gaps" and the total number of aberrant cells with or without "gaps") were statistically higher in the exposed group than in the reference group, except for the "gaps" or "acromatic lesions" (**Table III**). The types of CA found in the exposed population are shown in **figures 4** and **5**. Among all the CA registered, chromatidic aberrations were the most frequent type of CA observed in the exposed population.

Induction of CA in the exposed population: Our results are in agreement with other studies regarding that exposure to As via drinking water increases the frequency of CA in whole blood lymphocytes (Ostrosky *et al*. 1991, Huang *et al*. 1995, Gonsebatt *et al*. 1997, Basu *et al*. 2001, Mahata *et al*. 2003, Chackraborty *et al*. 2003). For example, Mahata *et al*. (2003) reported significant differences in all cytogenetic parameters analyzed, registering a percentage of aberrant cells with gaps of 0.80 ± 0.24 and a frequency of chromatidic aberrations per cell of 0.08 ± 0.002. These observations support our results (0.97 ± 0.01 and 0.09 ± 0.01, respectively) since As concentrations in drinking water (211.70 ± 15.28) were similar to those reported in this study.

Other studies about As in drinking water report higher frequencies of CA than the frequencies found in this study (Ostrosky *et al*. 1991, Gonsebatt *et al*. 1997, Liou *et al*. 1999, Maaki-Paakanien 1998).

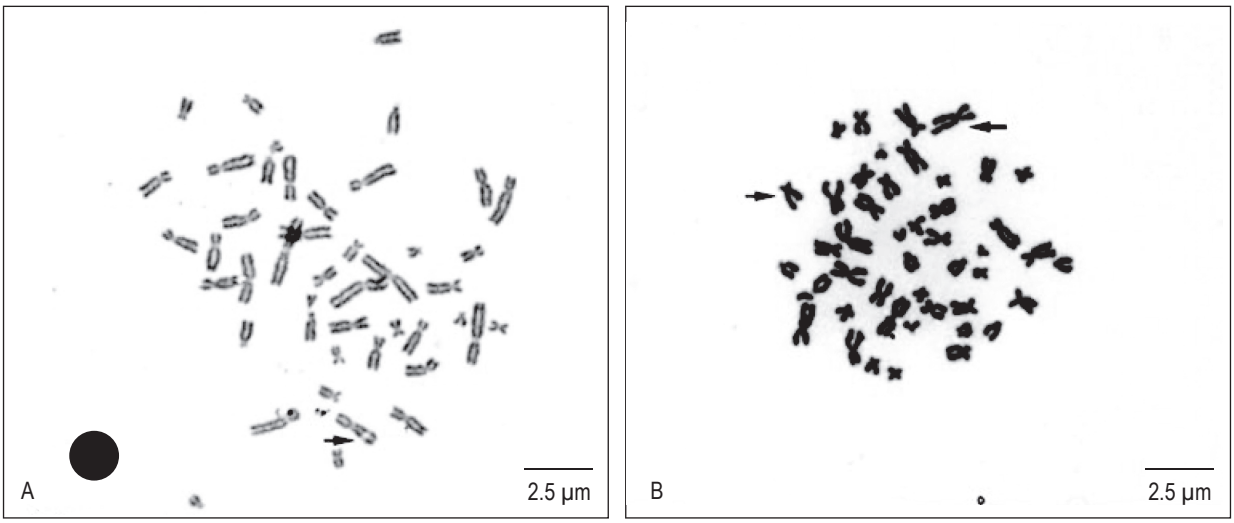

Fig. 4. Metaphase spreads of two exposed individuals. (A) "gap" and (B) two chromosomes with terminal or chromatidic deletions

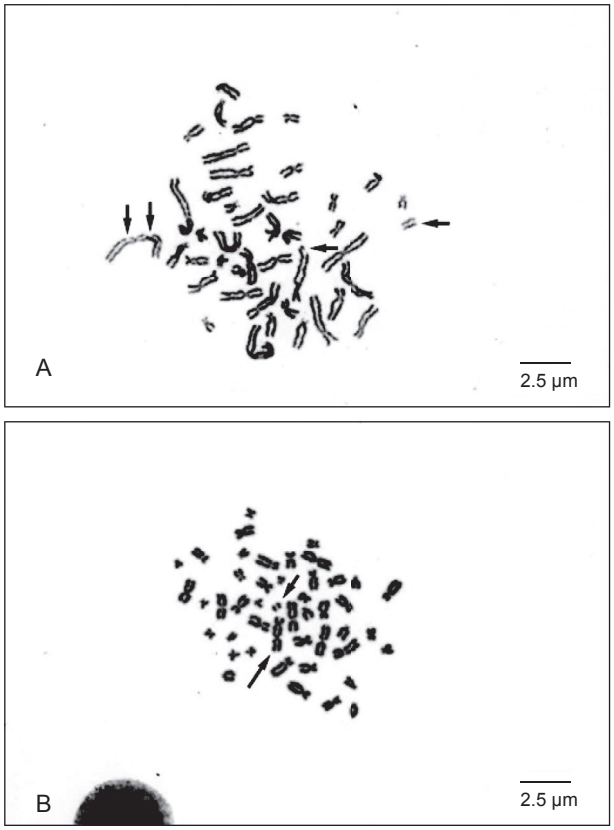

Fig. 5. Metaphase spreads of two exposed individuals. (A) two or more "gaps" in the same chromosome, an acentric fragment and an isochromatidic fragment. (B) dicentric chromosome and an isochromatidic fragment

These differences may be the result of various factors such as: different As water content, different genetic make-up of the populations involved like genetic polymorphisms (xenobiotic metabolizing enzymes, DNA repair and folate metabolism enzymes), exposure to other genotoxins, different nutritional status and life style factors etc. (Norppa *et al.* 2006). All these elements may contribute to the observed differences in the frequencies of CA.

From all the cytogenetic parameters analyzed in this study, the only one that did not showed significant differences between the exposed and the reference group was the induction of "gaps" or "acromatic lesions" ($t = 1.452$, $P > 0.05$; **Table III**). These observations may be related to the fact that "gaps" do not represent real chromatidic DNA lesions. Instead, they may represent decondensation of DNA fragments, sites of incomplete DNA synthesis, sites of improper DNA condensation or technical artifacts which may not represent real DNA lesions. Therefore, their frequency may be similar between exposed and reference individuals (Gonsebatt *et al*. 1997, Bender *et al*. 1998, Maaki-Pakaanien 1998, Basu *et al*. 2001, Mahata *et al*. 2003, Au and Salama 2005), and their biological importance is a matter of controversy (Natarajan *et al*. 2002, Savage 2004).

A great amount of evidence exists which demonstrates that As and their compounds (mostly trivalent) are capable of inducing CA (Basu *et al*. 2005, Norppa *et al*. 2006, Rossman and Klein 2011). Arsenic is considered a clastogen chemical compound, capable of producing DNA breaks. For an "S" dependent agent to produce CA it must produce SSB which during DNA repair or synthesis processes may be converted to Double Strand Breaks (DSB) and eventually to CA (Savage 2004, Rossman and Klein 2011). If DSB are not correctly repaired, they may result in mutation formation, chromosomic re-arrangements and oncogenic transformation.

Okui and Fujiwara (1986) and Lee-Chen *et al*. (1992) were among the first to report that base excision repair was altered in human fibroblast exposed to inorganic As. Also, Hartwig *et al*. (1997) reported that nucleotide excision repair as well as genomic global repair and transcription coupled repair mechanisms were altered in human fibroblast after exposing the cells to UV light and trivalent As. Hu (1998) explained these observations by demonstrating that phosphorylation patterns of proteins that are involved with ligase enzyme activities are altered. Another important observation was made by Huges in (2002), who detected that eukaryotic cells respond to DNA breaks by the activation of the poly-(ADP-ribose) polymerase enzyme which is involved in the repair of DSB. This enzyme contains two vicinal sulfhydryl groups where As may bind and alter its function (Hartwig *et al*. 2003, Beyersmann and Hartwig 2008).

Another mechanism by which As may induce CA is the generation of reactive oxygen species (ROS). Arsenic is capable of inducing oxidative damage in a great variety of cells, including human whole blood lymphocytes (Sampayo-Reyes *et al*. 2010, Wnek *et al*. 2011). Formation of ROS by As and its compounds may be the result of a decrease in mitochondrial membrane potential and intracellular glutathione concentrations, NADH oxidase activation, and by the oxidation of arsenite to arseniate (Del Razo *et al*. 2001). From all the effects produced by these mechanisms, the induction of genotoxic damage is the most important biologically since this would be the origin of the formation of CA, mostly chromatidic deletions that have been related to the formation of 8-oxo-dG, a lesion of maximal biological importance that when repaired, CA may be formed (Quian *et al*. 2003). Other studies that support the aforementioned

observations, have demonstrated that As lowers the intracellular levels of antioxidant enzymes such as: gluthatione, superoxide dismutase (SOD) and catalase, a fact that increases its clastogenic potential (Hey and Liu 1998, Quian *et al*. 2003).

Another indirect mechanism that may give rise to CA by As exposure, resides in the alteration of epigenetic regulation (Salnikow and Zhitkovich 2008). Pilsner *et al*. (2007) demonstrated that in individuals exposed to As via drinking water in Bangladesh, there was a positive relationship between the methylation levels in whole blood lymphocytes and As exposure. This effect was influenced by folate status among individuals, concluding that individuals with deficient folate intake presented higher levels of genomic methylation in whole blood lymphocytes in comparison with unexposed individuals. Taking into account these observations and the fact that Huautla inhabitants have nutritional and folate deficiencies (SSA 2003) we expect that they might be more susceptible to As effects which in turn, may elevate their CA frequency.

Relationship between CA frequency and As blood concentrations.

A positive and significant relationship was found between As blood concentrations and the frequency of all CA analyzed, except for the acentric fragments. Correlation coefficients (r) varied from 0.55 for the gaps to 0.85 for the total number of CA with gaps and the percentage of aberrant cells with gaps (**Table 4**). Among the types of CA analyzed, terminal deletions registered the highest correlation coefficient (r = 0.84). As many authors have recognized, this type of lesion is commonly found in lymphocytes from exposed populations to As in drinking water (Ostrosky *et al*. 1991, Huang *et al*. 1995, Gonsebatt *et al*. 1997, Basu *et al*. 2001, Mahata *et al*. 2003, Chackraborty *et al*. 2006). This is because As is considered as an "S" phase dependent agent, which means that CA are produced during the "S" or "G2" phase of the cell cycle. In whole blood lymphocytes (G0) most of the chemical agents as in the case of As, induce chromatidic type aberrations, these insults will only be reflected if these cells are stimulated *in-vitro*. However, if these cells carry unrepaired or misrepaired lesions they will result in the formation of CA during DNA replication *in-vitro*. Chromatidic aberrations, which are characteristic of the "S" phase dependent chemical agents, indicate that the formation of CA is a post-replicative event (Savage 1990).

The types of CA that presented the lowest correlation coefficient were the isochromatidic fragments and gaps (r = 0.55). These lesions were the least frequent in the exposed individuals in comparison with deletions and acentric fragments. This is the first study that reports a positive relationship between blood As concentrations and different types of CA from the same individuals. Again, our results provide evidence that As whole blood concentrations can be a useful biomarker of exposure that correlates positively with different types of genotoxic damage.

TABLE IV. REGRESSION ANALYSES TO DETERMINE THE RELATIONSHIP BETWEEN TOTAL BLOOD ARSENIC CONCENTRATIONS IN THE EXPOSED POPULATION AND THE INDUCTION OF THE DIFFERENT TYPES OF CHROMOSOME ABERRATIONS

	R	R^2	$F_{1,40}$	P
Gaps	0.55	0.34	10.68	< 0.001
Terminal deletions	0.84	0.70	50.03	< 0.001
Isochromatidic fragments	0.55	0.30	8.99	< 0.001
T.N.C.A. with gaps	0.77	0.59	30.55	< 0.001
T.N.C.A. without gaps	0.85	0.72	53.92	< 0.001
% A.C. without gaps	0.64	0.41	14.74	< 0.001
% A.C. with gaps	0.85	0.33	10.57	< 0.01

T.N.C.A = Total number of chromosome aberrations. A.C. = aberrant cells

Alcohol intake, smoking habit and age effects on the induction of single strand breaks and chromosome aberrations

In the reference group, no effect of age, drinking habits or smoking status on the induction of SSB or the frequency of CA was observed. In contrast, the exposed population registered a significant effect of drinking habits on the induction of SSB ($F_{3,18}$ = 5.391, $P < 0.05$), but not age or smoking habits. For the exposed population no effect of age, drinking habits or smoking status on the frequency of CA induction was observed.

When assessing genotoxic damage in human lymphocytes, we should take into account some factors that may influence DNA damage levels, such as smoking status, alcohol intake, age and gender. In our study no correlation between age and smoking on the levels of DNA damage (SSB and CA) was observed, this may be due to higher levels of DNA damage registered in this particular population that could be masking the effect of lifestyle factors such as age and smoking habits. The individuals sampled that are currently smokers are light smokers. In most cases, the correlation between smoking and genotoxic damage appears to be positive when the individuals

are heavy smokers (King *et al*. 1997, Pitarque *et al*. 1999, Hyland *et al*. 2002, Fracasso 2006).

There was a significant effect of drinking habits on DNA damage levels (SSB) in the exposed population, it is noteworthy to mention that this particular population has heavy drinking status, on average each individual drinks two or three alcohol drinks daily. Various studies have demonstrated a relationship between the induction of SSB and alcohol intake. Alcohol consumption induces reactive oxygen species generation which in turn causes SSB that are detected with the comet assay (Navasumrit *et al*. 2000, Nomura *et al*. 2001, Pöschl and Seitz 2004).

Overall, this study showed that As levels in drinking water of Huautla, Morelos, Mexico are above the national and international standards from diverse regulatory organizations. As a consequence, Huautla inhabitants are in chronic and constant exposure to high levels of As in drinking water, representing another problem of hydroarsenisism in Mexico. Huautla settlers may be at risk of developing As-related diseases, a fact that urgently needs further evaluation. Furthermore, total As blood concentrations from the exposed individuals, correlates positively with the induction of SSB and different types of CA. Hence, we provide evidence that supports the use of total As blood concentrations as an internal biomarker of exposure in human populations. Particularly, in subjects with altered kidney function as in the case of chronic diseases such as Type 2 diabetes mellitus and hypertension, because these individuals may differ in the way they metabolize As (Del razo *et al.* 2011). A fact that would make difficult the measurement and normalization of As urine levels in these individuals. In conclusion, blood As is not the ideal exposure biomarker but it could be interesting to develop its use in the analysis of the early biological effects of As in populations with these health conditions.

ACKNOWLEDGMENTS

The authors thank Elgar Castillo Mendoza and Armando Zepeda for their technical assistance. This project was supported in part by Consejo Nacional de Ciencia y Tecnología (CONACyT) scholarship for P.M.G.

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